Histol Histopathol (2008) 23: 1453-1463

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Morphological effects of oestradiol-17ß, and selective oestrogen receptor α and ß agonists on luteinising hormone-secreting cells in tamoxifen-treated ovariectomised rats

José C. Garrido-Gracia[1], Ana Gordon[1], Rafaela Aguilar[1], José G. Monterde[2], Alfonso Blanco[2], Juana Martín de las Mulas[2] and José E. Sánchez-Criado[1]

[1]Departments of Cell Biology, Physiology and Immunology and [2]Comparative Pathology, University of Córdoba, Spain

*JCGG and AG equally contributed to this work.

Summary. To investigate the role played by the different rat gonadotroph oestrogen receptor (ER) pools in the effects of oestradiol-17ß (E2) on gonadectomy cells, two-week ovariectomised (OVX) rats were used. The basic experimental group of rats was injected with 3 mg of the selective ER modulator tamoxifen (TX) on days 15-20 after OVX. Groups of TX-treated OVX rats were additionally injected on days 18-20 after OVX with 10 µg oestradiol benzoate (EB), 1 mg of the selective ERα agonist propylpyrazole triol (PPT), or 1 mg of the selective ERß diarylpropionitrile (DPN). Negative and positive control groups were OVX rats injected over six days after OVX with 0.2 ml oil and EB, respectively. On day 21 after OVX, anterior pituitary glands were dissected out and divided into halves. One hemipituitary was processed for light microscopy and immunocytochemistry for ßLH subunit and progesterone receptor (PR), and the other hemipituitary for ultrastructural evaluation. Results showed that: gonadotrophs were the only pituitary cell type expressing PR; treatment with TX alone shrunk gonadectomy cells and induced both reorganization of membrane-enclosed intracellular organelles and PR expression, and treatment with DPN or EB, but not PPT, reduced the agonistic morphological effects of TX. Considering that TX activates nuclear ERα, the results indicate that activation of nuclear ERα is determinant for the reversal effects of E_2 on gonadotrope morphology and PR expression, and the simultaneous activation of ERß modulates the action of ERα in an inhibitory fashion.

Offprint requests to: José Carlos Garrido Gracia, Sección de Fisiología, Facultad de medicina, Avda Menendez Pidal s/n, 14004, Cordoba, Spain. e-mail: bc2gagrj@uco.es

Key words: Gonadotroph morphology, Selective ER agonists, Tamoxifen, ERα and ß, Progesterone receptor

Introduction

The main steps of the biosynthesis/secretory luteinising hormone (LH) cycle in the rat gonadotroph are: 1) transcription of the genetic information into RNAs; 2) translation via mRNAs of this information in the cytoplasm; 3) posttranslational modifications in the membrane-enclosed intracellular organelles, rough endoplasmic reticulum (RER), Golgi complex and secretory granules; and 4) discharge of LH from the secretory granules by regulated exocytosis (Pierce, 1988). Any step in this process is a locus for the physiological oestradiol-17ß (E_2) control of LH synthesis and secretion acting on the complete oestrogen receptor (ER) orchestra (Fink, 1988, 1995) (nuclear and membrane ERα and ß isoforms) (Nishihara et al., 2000; Petersson and Gustafsson, 2001). Because of the continuous presence of E2 over the 4-day oestrous cycle of the rat (Smith et al., 1975), the E2-dependent morphological changes in gonadotrophs are not well defined (Tougard and Tixier-Vidal, 1988). Thus, the decrease in E_2 levels after the LH surge in pro-oestrous afternoon (Freeman, 1988) causes subtle ultrastructural modifications in gonadotrophs (Moriarty, 1975), a slight increase in size (Morris et al., 1986), loss of progesterone receptors (PR) expression and absence of the PR-dependent LH releasing hormone (LHRH) self-priming (Castro-Vazquez and McCann, 1975; Sánchez-Criado et al., 2005a), the property of LHRH that increases gonadotroph responsiveness to itself (Fink, 1995).

The two-week ovariectomised (OVX) rat treated over three days with E2 mimics the endocrine events of intact pro-oestrous rats (Smith et al., 1975; Freeman, 1988), and exhibits the expected LH surge (Legan and Tsai, 2003). Because of these facts, the long-term OVX rat is a useful model to study gonadotroph function (Sánchez-Criado et al., 2005a,b, 2006a). Gonadotrophs, but no other pituitary cell type, of both OVX rats (Genbacev and Pantic, 1975) and ERα-knockout mice (Pelletier et al., 2003), are hypertrophic (gonadectomy cells, OVX cells) with typical ultrastructural disorganization of intracellular organelles (Garner and Blake, 1981; Childs, 1986; Sánchez-Criado et al., 2006a) lacking PR expression (Sánchez-Criado et al., 2004, 2005b). Gonadotrophs are the only type of pituitary cells that express PR (Fox et al., 1990; Bellido et al., 2003), and the presence of PR is essential for the full release of the preovulatory LH surge (Chappell et al., 1999). E_2 treatment reverses the effects of OVX on gonadotroph size and morphology, PR expression and secretion of LH (Genbacev and Pantic, 1975; Sánchez-Criado et al., 2004, 2005a). Activation of the ERα isoform alone using the selective agonist propylpyrazole triol (PPT) (Stauffer et al., 2000) induces gonadotrope shrinkage and reverses the effects of OVX in all parameters of LH secretion (negative and positive feedbacks in the rat) (Sánchez-Criado et al., 2004). On the other hand, activation of the ERß, isoform alone using the selective agonist diarylpropionitrile (DPN) (Meyers et al., 2001) in OVX rats induces PR expression without any secretory activity (Sánchez-Criado et al., 2004, 2006a).

Tamoxifen (TX) is a selective ER modulator (SERM) (Tzukerman et al., 1994; Hall and McDonnell, 1999) that, in the absence of E_2, exhibits at the rat gonadotroph level nuclear ERα-like responses similar to those exhibited by the selective agonist PPT: induction of ERα and PR expression and GnRH self-priming (Sánchez-Criado et al., 2002, 2005a, 2006b; Alonso et al., 2006; Aguilar et al., 2006; Garrido-Gracia et al., 2007). Moreover, treatment with the *pure* anti-E RU58668 (Bellido et al., 2003) or the potent ERα-selective antagonist (220-fold more affinity for ERα than for ERß) methyl-piperidino-pyrazole (Sun et al., 2002) block all agonistic actions of TX on the rat gonadotroph (Garrido-Gracia et al., 2007). Finally, TX seems to have no functional affinity for membrane ERα in the rat gonadotroph (Sánchez-Criado et al., 2005b). Based on these studies in OVX rats we have proposed that E_2 action on the gonadotroph in the rat is exerted at the nuclear ERα pool, and that this action is modulated by E_2 effects on both nuclear ERß and surface ERα. E-dependent gonadotroph shrinkage (Sánchez-Criado et al., 2006a) and PR expression (Scott et al., 2002) are the morphological basis for our proposition (Garrido-Gracia et al., 2007), as both of them are critical for the induction of LHRH self-priming, and hence preovulatory LH secretion in the rat. However, the probable modulatory activity of ER isoforms and sites (Levin, 2005) on gonadotroph morphology and PR expression in OVX rats has not been analysed. In the present study we investigated, in OVX rats undergoing TX-activated nuclear ERα in the gonadotroph, the effects of EB, PPT and DPN upon the size, structural and ultrastructural characteristics and PR expression of gonadectomy cells.

Materials and Methods

Animals and treatments

Adult female Wistar rats weighing 190-210 g were used. Rats were housed under a 14:10 h light-dark cycle (light on at 0500 h) and 22±2°C room temperature, with *ad libitum* access to rat chow devoid of oestrogenic activity (A04, Scientific Animal Food & Engineering, Augy, France) and tap water. All rats were ovariectomised (OVX) under ether anaesthesia at random stages of the oestrous cycle and assigned to experimental groups (4 rats each) 14 days later. All four experimental groups received daily sc injection of 3 mg tamoxifen (TX; Sigma Chemical Co., St. Louis, MO, USA) on days 15-20 after OVX. Three groups of TX-treated rats were additionally treated on days 18-20 after OVX with 10 µg oestradiol benzoate (EB; Sigma), 1 mg of the selective ERα propylpyrazole triol (PPT), or 1 mg of the ERß agonist diarylpropionitrile (DPN) (Tocris Cookson Ltd, Avonmouth, UK). PPT has a 400-fold preference for ERα and does not activate ER, (Stauffer et al., 2000) and DPN has a 70-fold higher binding affinity for ERß than for ERα (Meyers et al., 2001). Negative and positive control groups were OVX rats injected on days 15-20 after OVX with 0.2 ml olive oil and 10 µg EB, respectively. Dosages of ER ligands used in the present experiments were based on previously published studies (Schuiling et al., 1987; Bellido et al., 2003; Sánchez-Criado et al., 2005a,b, 2006a,b; Arreguin-Arevalo et al., 2007; Garrido-Gracia et al., 2007), including dosage-dependency (Sánchez-Criado et al., 2004) and the ER ligand ability to induce vaginal cornification. All rats were decapitated on the morning of day 21 and the lobes of their anterior pituitaries sagitally separated. One hemipituitary was processed for light microscopy and immunohistochemistry of PR and ßLH subunit, and the other hemipituitary for electron microscopy. For comparative purposes 4 pituitaries from intact cycling rats in pro-oestrus were similarly processed. All experimental protocols were approved by the Ethical Committee of the University of Córdoba, and experiments were performed in accordance with the rules of laboratory animal care and international law on animal experimentation.

Tissue preparation for light and electron microscopy

For light microscopy, hemipituitaries were fixed in 10% buffered formalin and embedded in paraffin-wax. For electron microscopy, small fragments from each of the remaining hemipituitaries were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4)

followed by post-fixation in 1% osmium tetroxide in distilled water for 2 h. Tissue fragments were then embedded in Araldite and cut at 0.1 µm with an ultramicrotome. Current ultrastructural characteristics of membrane-enclosed intracellular organelles (RER, Golgi complex and secretory vesicles) in gonadotrophs (Garner and Blake, 1981; Childs, 1986; Sánchez-Criado et al., 2006a) were evaluated.

Immunohistochemical detection of progesterone receptor (PR) in ßLH subunit expressing cells

Pituitaries from OVX rats injected with oil (negative control group) or ER ligands were studied for the simultaneous immunohistochemical co-localization in single histological sections of LH and PR. This immunohistochemical study was performed on dewaxed and rehydrated 3-µm thick tissue sections of formalin-fixed, paraffin-embedded tissue samples. The expression of LH was analysed using the commercial polyclonal rabbit anti-human beta-luteinising hormone (ßLH) antibody (AbD Serotec, Oxford, Oxon, UK) diluted 1:10 for 18 hours at 4°C, and the peroxidase anti-peroxidase (PAP) technique. Swine anti-rabbit IgG diluted 1:20 and rabbit PAP diluted 1:50 were applied for 30 minutes each at room temperature and the immune reaction was developed with the chromogen DAB. The expression of PR was analysed using the commercial mouse monoclonal anti-human PR antibody clone PR10A9, raised against the recombinant hormone-binding domain of human PR located on the C-terminal domain of PR (Immunotech, Marseille, France), diluted 1:15000 for 18 hours at 4°C, and the streptavidin-biotin-alkaline phosphatase (SABAP) complex technique (StrAviGen Super Sensitive, Biogenex, San Ramon, CA, USA). The latter was applied following the recommendations of the manufacturer, and the immune reaction was developed with chromogen fast red. Corresponding reagents for each individual technique (anti- ßLH and anti-PR antibodies; swine anti-rabbit IgG and biotinylated goat anti-mouse IgG, respectively; and the PAP and SABAP complexes, respectively) were mixed (1:1) and used at twice their standardized optimal dilutions. Cells with co-localization of ßLH and PR displayed brown cytoplasm and red nucleus, respectively. Tissue sections from similarly-processed samples of pituitaries from intact cycling rats in pro-oestrus, rat uterus and human breast carcinoma were used as positive controls. Incubation with non-immune rabbit serum and mouse IgG2a (Dako, Barcelona, Spain) at the same dilution as the specific primary antibodies in tissue sections of the cases under study was used as a negative control. Slight nuclear counterstaining was performed with Mayer's haematoxylin in all cases. The amount of cells immunoreactive with ßLH antibody was expressed as the percentage of positive cells counted in 5 fields/section in three different consecutive tissue sections taking one every four at a magnification of 40 times (about 240 pituitary cells/field) in each pituitary.

Identification and classification of gonadotrophs

At light-microscopy all pituitary cells expressing LH were identified as gonadotrophs and further classified into two subgroups: gonadotrophs with PR expression and hypertrophied gonadotrophs without PR expression.

At electron-microscopic level gonadotrophs were identified because of the peculiarities of both the Golgi complex (always close to the nucleus) and the cisternae of RER (with proteinaceus content all over the cytoplasm) (Tougard and Tixier-Vidal, 1988; Sánchez-Criado et al., 2006a). In addition, and taking into account: i) nuclear size; ii) cytoplasmic vacuolization and presence of cisternae; iii) functional appearance of RER, Golgi complex and secretory granules; and iv) distribution and proximity to vascular vessels, gonadotrophs were classified into four subtypes (see the results section). Gonadotrophs were distinguished from other types of basophilic cells in the pituitary because both of the size and the location of their secretory granules (Gartner and Hiatt, 2001).

Morphometric study

The size of gonadotrophs (μm^2 in area) was evaluated in digitalized images of immunostained tissue sections by using the VISILOG 5 program as described previously (Blanco et al., 2001; Sánchez-Criado et al., 2006a). Twenty digitalized fields per hemipituitary were evaluated. Each field was digitalized at 40x magnification. The four types of gonadotrophs identified at electron-microscopic level described in the results section were counted and expressed as a percentage relative to the total number of gonadotrophs.

Statistics

Statistical analysis was performed by ANOVA to test the existence of significant differences among groups. When significant differences existed, it was followed by the Student-Newman-Keuls multiple range test for intergroup comparison. Significance was considered at the 0.05 level.

Results

Colocalization of PR and ßLH subunit in rat pituitary cells

Immunoreactive products to PR and ßLH antibodies were observed in the nucleus and cytoplasm of anterior pituitary cells, respectively. In pituitaries from cycling rats in pro-oestrus, all nuclear PR-positive cells expressed cytoplasmic ßLH (Fig. 1A,B). In pituitaries from OVX rats injected with oil, large, rounded cells with eccentric nuclei (gonadectomy cells) were the only type of cells expressing ßLH, while PR expression was absent in these as well as any other type of anterior pituitary cells (Fig. 1C,D). In pituitaries from ER ligands treated animals there were ßLH-positive cells with and

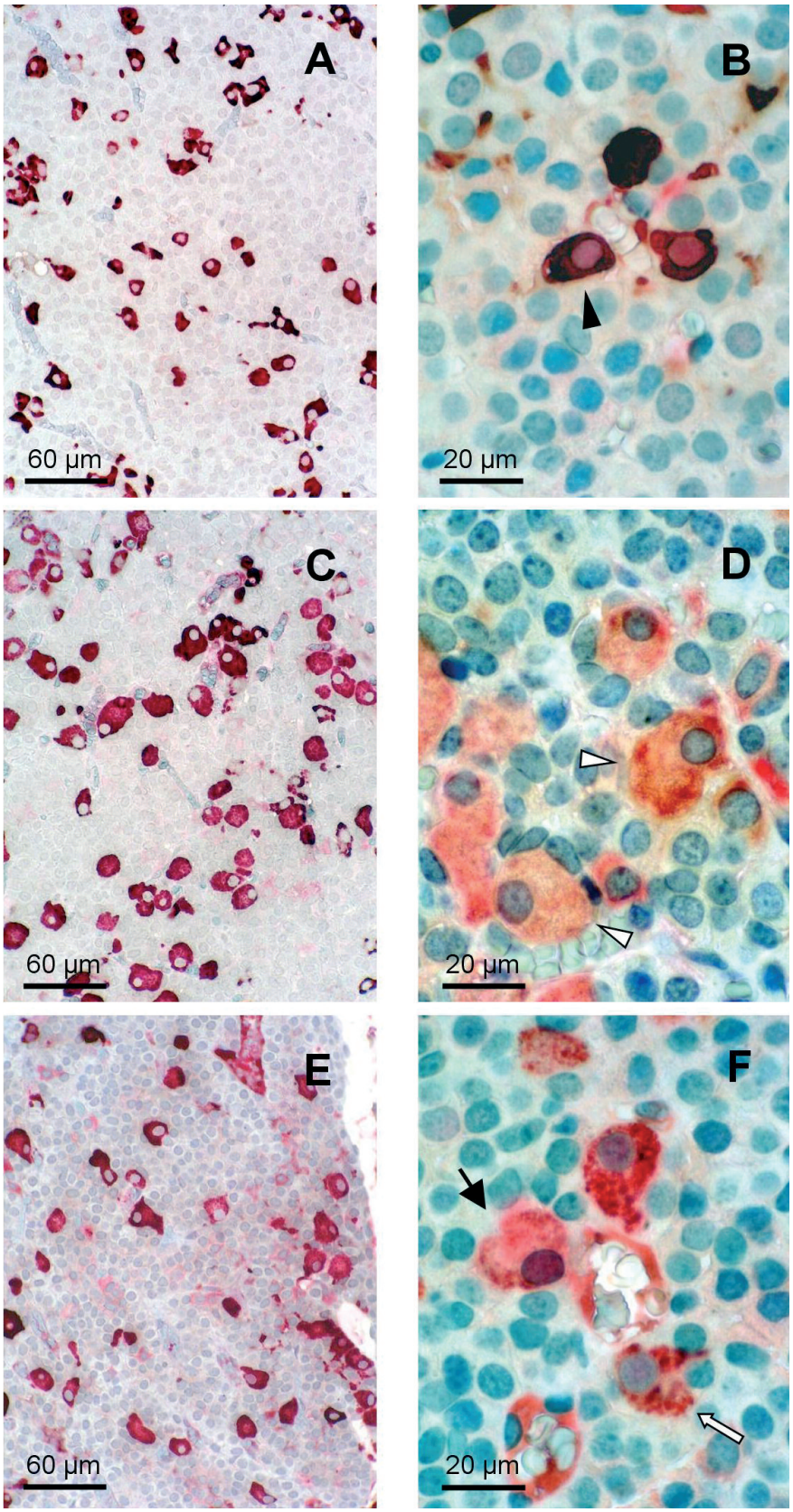

Fig. 1. Micrographs of pituitaries from cycling rats in pro-oestrus **(A, B)**, and from two-week ovariectomized (OVX) rats injected over six days (15-20 after OVX) with 0.2 ml oil **(C, D)** or 3 mg of tamoxifen (TX) **(E, F)** showing the immunohistochemical co-localization of ßLH and PR. Immunoreactive products to ßLH antibody were found in the cytoplasm of cells as a deep brown **(A, B)** to light **(C, D)** or medium brown **(E, F)** reaction, while immunoreactive products to PR antibody were found in the nuclei as a red reaction. The brown cytoplasmic reaction was more or less masked by the unspecific backgroung staining due to the chromogen fast red, also observed as a red staining of serum **(F)**. In pituitaries from cycling rats in pro-oestrus **(A, B),** both ßLH (red-brownish cytoplasm) and PR (red nucleus) were always expressed in the same cells (black arrowhead). In pituitaries from two-week OVX rats injected with oil **(C, D)**, ßLH was observed in the cytoplasm of large, rounded cells with eccentric nuclei (gonadectomy cells) (open arrowheads), while PR was not observed in any type of anterior pituitary cell. In pituitaries from two-week OVX rats injected with TX **(E, F)** there were ßLH-positive cells of different sizes with (black arrow) and without (open arrow) PR expression. PAP and SABAP immunohistochemical techniques.

without PR expression, although in different proportions: the largest gonadectomy cells lacked PR expression while the smallest did express PR (Fig. 1E,F).

Effects of ER ligands treatment on gonadotrope size and PR expression in OVX rats

Gonadotrophs (ßLH positive cells) accounted for about 9% of all pituitary cells. This percentage of LH-secreting cells was not affected by any ER ligand treatment. However, ER ligand treatments induced changes in the percentage and size of gonadotrophs with and without PR expression (Fig. 2). Thus, pituitaries from OVX rats injected with oil (negative control group) had hypertrophied gonadotrophs without PR expression exclusively, while those of OVX rats treated with ER ligands exhibited both hypertrophied gonadotrophs without PR expression and gonadotrophs variably shrunk with PR expression (Fig. 2). EB injections to OVX rats (positive control groups) halved the numbers of gonadotrophs lacking PR expression and reduced their size in favor of shrunk gonadotrophs expressing PR (Fig. 2, 3). This oestrogenic effect of the cognate ligand was also exhibited by the pituitaries from OVX rats treated with TX alone (Fig. 2). The additional treatment of TX-injected rats with EB or DPN, but not with PPT reduced both TX-induced gonadotroph shrinkage and TX-elicited PR expression (Fig. 2). The relationship between size and PR expression in gonadotrophs is

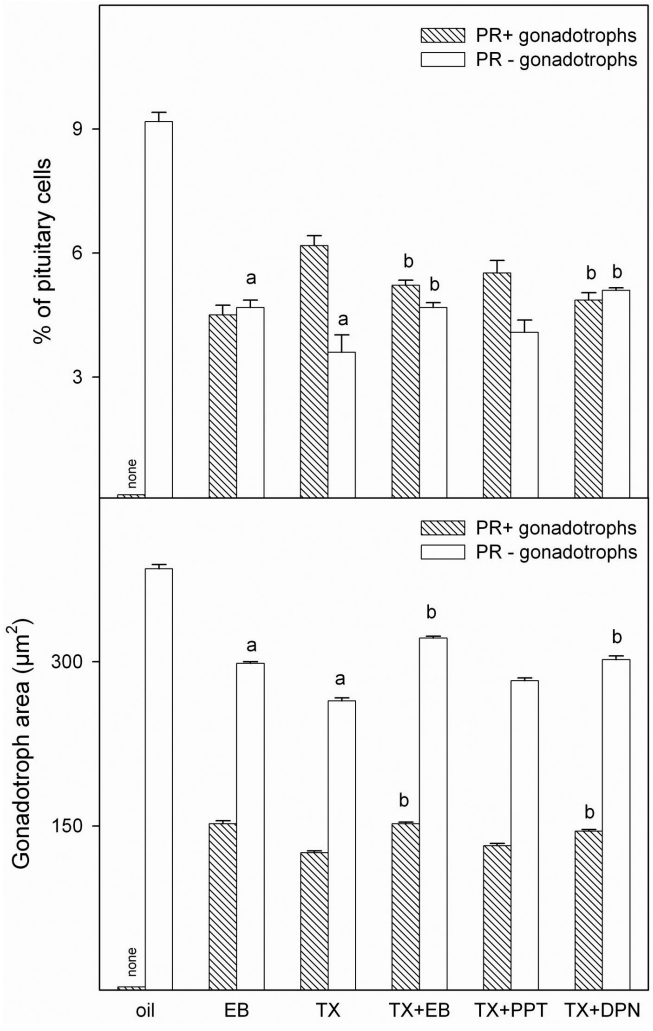

Fig. 2. Percentage of pituitary cells (upper panel) and gonadotroph area (lower panel) expressing (+) or not expressing (-) progesterone receptor (PR) from two-week ovariectomized (OVX) rats on day 21 after OVX. Rats were injected over six days (15-20 after OVX) with: 0.2 ml oil (negative control group); 10 µg of oestradiol benzoate (EB) (positive control group); and 3 mg of tamoxifen (TX) alone, or TX-injected (days 15-20) OVX rats given 10 µg EB (TX+EB); 1 mg PPT (TX+PPT); and 1 mg DPN (TX+DPN) on days 18-20. a: P<0.05 vs. oil; b: P<0.05 vs. TX. ANOVA followed by the Student-Newman-Keuls multiple range test. Values are means ± SEM.

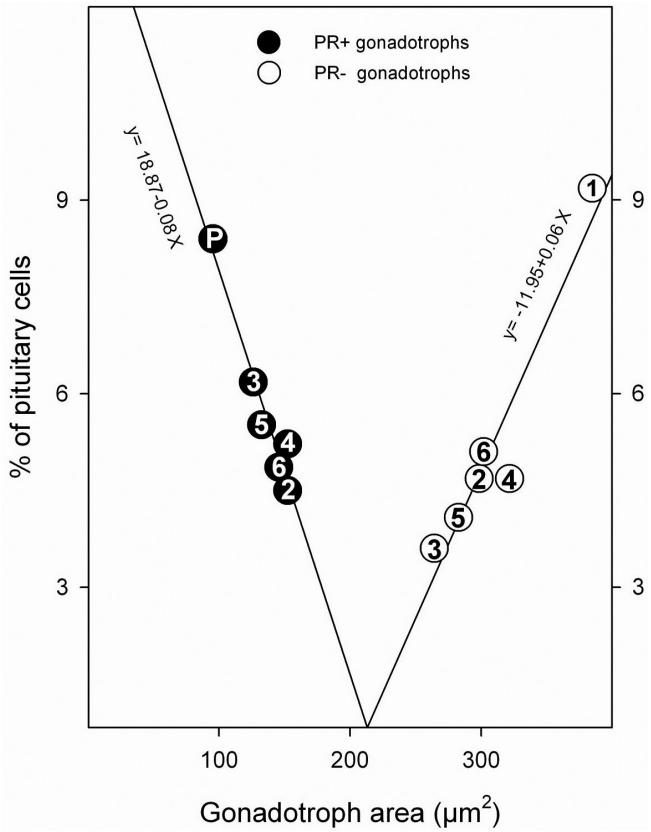

Fig. 3. Relationship between gonadotroph size and percentage of pituitary cells expressing (+) or not expressing (-) progesterone receptor (PR). 1: OVX+oil; 2: OVX+EB; 3: OVX+TX; 4: OVX+TX+EB; 5: OVX+TX+PPT; 6: OVX+TX+DPN; P: percentage and size of gonadotrophs from 4 intact cyclic rats in pro-oestrus. See legend of figure 2 for details of treatments. Values are the mean of those represented in figure 3. Note that the critical size for the acquisition of the ability to express PR is about 200 µm^2.

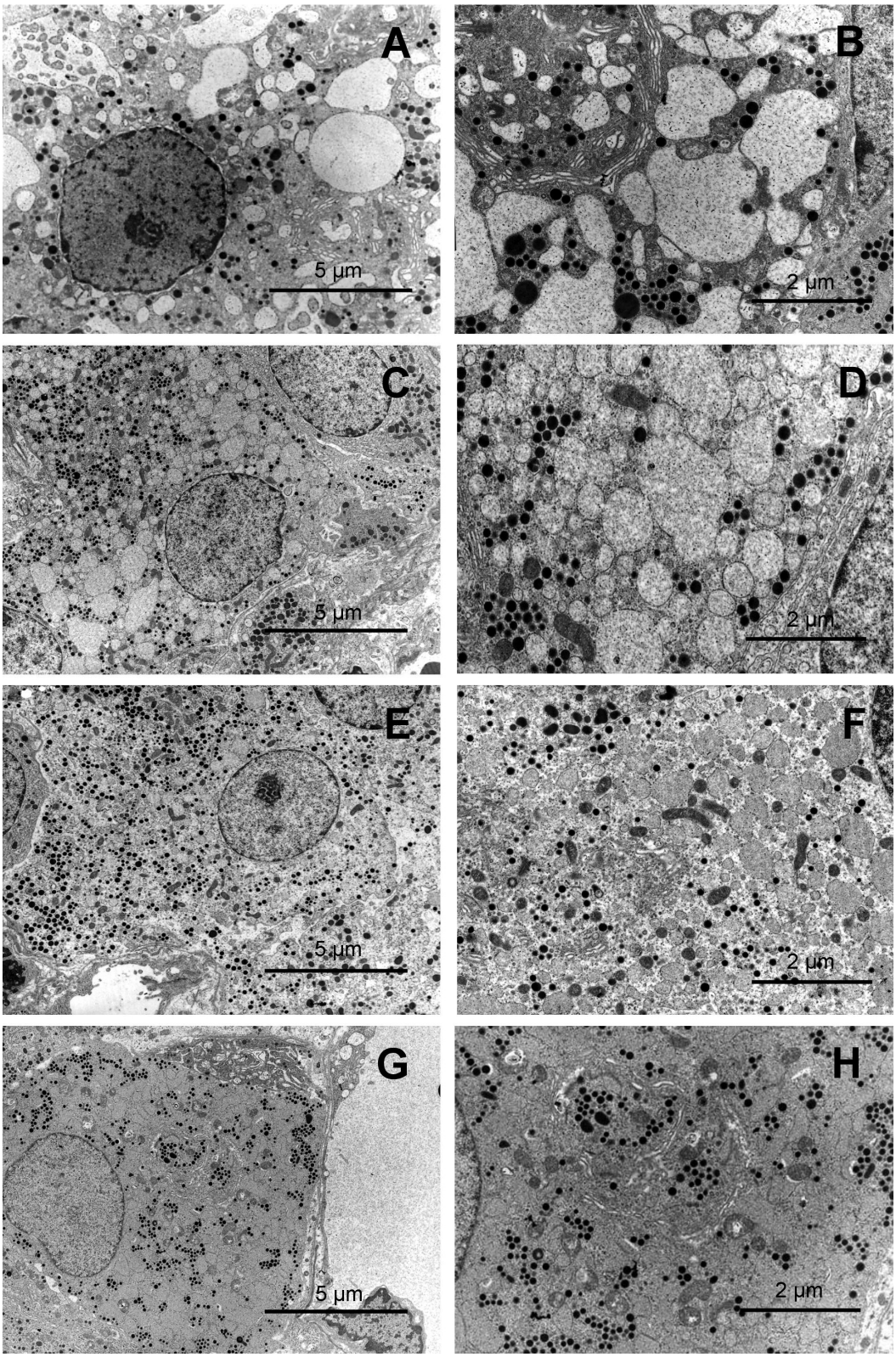

Fig. 4. Representative examples of gonadotroph subtypes 1 **(A, B)**, 2 **(C, D)**, 3 **(E, F)** and 4 **(G, H)** on day 21 after ovariectomy (OVX) in rats injected daily from day 15 to day 20 after OVX with: 0.2 ml oil **(A, B)**; 10 µg oestradiol benzoate (EB) **(C, D)**; 3 mg tamoxifen (TX) **(G, H)**; and with both TX and the ER, selective agonist DPN given during three days (18-20 after OVX) **(E, F)**. **A.** Several vacuoles of different sizes occupy the cell cytoplasm. The nucleus is large, with condensed chromatin and vacuolization of the nuclear envelope. **B.** The dilated RER cisternae from large, empty vacuoles. **C.** Abundant pleomorphic RER cisternae with scant proteinaceous content are seen. The nucleus appears normal. **D.** In addition to abundant and pleomorphic RER cisternae with scant content, a small Golgi complex with normal saccules is noted. Numerous cisternae with homogeneous content are seen. **E.** Numerous RER cisternae with homogeneous content are found. The nucleus appears normal and secretion granules are abundant. **F.** Large, widely separated RER cisternae with dense content. A small Golgi complex with dilated saccules can be seen. **G.** Gonadotroph subtype 4 close to a blood capilary vessel. The RER is well developed, with tightly packed, small cisternae with dilated lumen and proteinaceous content. The Golgi complex is large, and numerous secretion granules are distributed in clusters throughout the cell cytoplasm as well as marginalized close to a capillary vessel. **H.** Magnification of the Golgi complex and RER from G.

shown in Fig. 3. Corresponding data of gonadotrophs from intact pro-oestrous rats have been included for comparison purposes. In the present experiments, gonadotrophs measuring less than 200 μm^2 always expressed PR, while those measuring more than 200 μm^2 did not (Fig. 3).

Ultrastructural features of gonadotrophs in OVX rats treated with different ER ligands

Ultrastructurally, four different subtypes of gonadotrophs were distinguished in OVX rats. Gonadotrophs subtype 1 were isolated cells with low RER and Golgi complex activity (Fig. 4A,B). Thus, empty RER cisternae were dilated and fused, giving rise to vacuoles of different sizes occupying the cell cytoplasm, while Golgi complex membranes were either absent or scarce. The nucleus was enlarged with condensation of chromatin and dilatation of the perinuclear space. Isolated secretory granules had signs of degeneration. Gonadotrophs subtype 2 (Fig. 4C,D) were isolated cells and had an extensive cytoplasmic network of irregular, pleomorphic RER cisternae with scant uniform content. The Golgi complex was small and had normal-appearing flattened saccules, while the nucleus, although enlarged, lacked chromatin condensation and was smaller than that of gonadotrophs subtype 1. Secretory granules had a normal appearance but an abnormal distribution. Gonadotrophs subtype 3 (Fig. 4E,F) were found both as isolated cells and forming small groups of cells. Gonadotrophs subtype 3 had large, widely separated RER cisternae with electrodense content, small, normal-appearing Golgi complex, normal nuclei and secretory granules with a normal appearance, but still with irregular distribution. Gonadotrophs subtype 4 (Fig. 4G,H) were found in groups of four to five small cells close to blood capillaries (Fig. 4G). Their cytoplasm was almost completely occupied by a dense network of small, uniform cisternae with dense proteinaceous content. The Golgi complex was composed of several units normally distributed around the nucleus, and secretion vesicles in various stages of condensation and maturation were abundant. The nucleus had normal size and appearance and electrodense secretory granules were clustered and marginalized between RER cisternae. These gonadotroph subtypes represent various ultrastructural steps of the effect of ER activation, ranging from complete absence (subtype 1) to fully activated ER (subtype 4).

OVX rats injected with oil (negative control group) had a similar proportion of gonadotrophs subtypes 1 and 2 exclusively, while OVX rats treated with ER ligands presented varying proportions of gonadotrophs subtypes 2, 3 and 4 (Fig. 5). TX-injected OVX rats, but not EB-injected OVX rats (positive control group) presented a significant decrease in the percentage of gonadotroph subtype 2 (38±4 vs. 52±3) in favor of subtype 4 (48±3

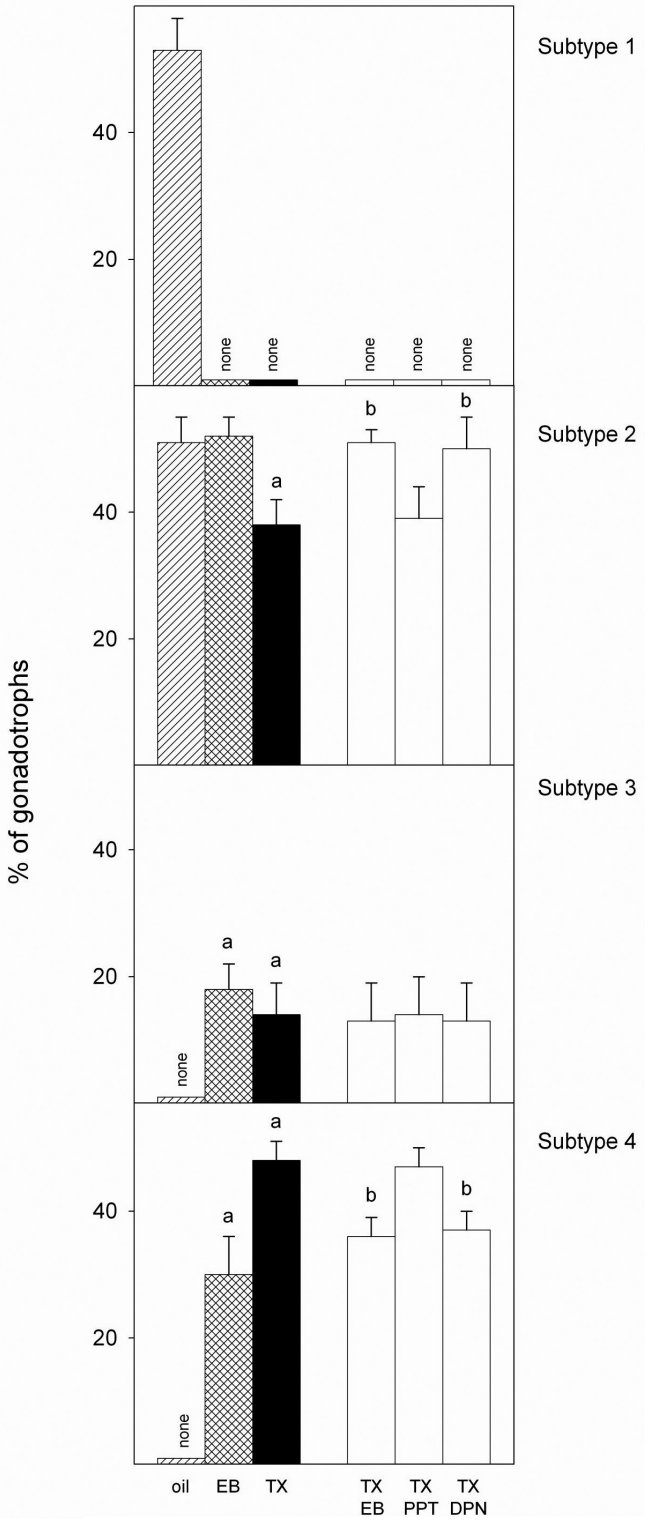

Fig. 5. Percentages of each gonadotroph subtype found in the pituitaries from two-week ovariectomised rats treated with oil, EB, TX, TX+EB, TX+PPT and TX+DPN. The total number of gonadotrophs analysed was about 50/group. See legend of figures 2 and 4 for details of treatments and gonadotroph subtype classification, respectively. a: $P<0.05$ vs. oil; b: $P<0.05$ vs. TX. ANOVA followed by the Student-Newman-Keuls multiple range test. Values are means ± SEM.

vs. 30±6) with respect to the negative and the positive control groups, respectively. Finally, the effect of the different ER ligands used on TX-injected OVX rats differed. While PPT had no effect, both dosages of EB and DPN blunted the effects of the six-day TX treatment on gonadotrophs subtypes 2 and 4, but had no effect on the percentage of gonadotroph subtype 3, which appears to represent a transitional stage from subtype 2 to subtype 4 (Fig. 5).

Discussion

Accumulated evidence from recent physiological experiments indicate that TX acts, in the absence of E_2, as a nuclear ERα agonist in the gonadotroph of the rat (Sánchez-Criado et al., 2002, 2004, 2005a,b, 2006a,b). These findings agree with those observed in transfected cells expressing ERα alone, in which TX always behaves as an oestrogen agonist (Hall and McDonnell, 1999). This *in vitro* evidence have been further corroborated in *in vivo* system conditions (Garrido-Gracia et al., 2007). Therefore, it seems convincing that the agonist actions of TX in the rat gonadotroph are exerted mainly at the nuclear ERα level. The results of the present study indicate that nuclear ERß, but not extranuclear ERα, modulates the morphological action of the predominant nuclear ERα on the gonadotrophs of long-term OVX rats. Thus, activation of nuclear ERα with the SERM TX reversed the effects of OVX-induced E_2 withdrawal on gonadotroph size and morphology and induced PR expression. Simultaneous activation of nuclear ER, with either its selective agonist DPN, or the cognate ligand EB, blunted the morphological effects putatively elicited by activation of nuclear ERα with TX. On the contrary, the selective ERα agonist PPT failed to affect TX agonistic actions on gonadotrophs.

The percentage of gonadotrophs identified in the present study was similar to those found in previous studies of the rat pituitary (Childs et al., 1982; Childs, 1986; Fellmann et al., 1982; Cornea et al., 1998; Sánchez-Criado et al., 2005a, 2006a), and was not affected by any ER ligand treatment. It is known that two weeks after OVX, the hypertrophied gonadotrophs or gonadectomy cells still express immunoreactive oestrous cycle-related nuclear ERα, constitutive nuclear ERß isoforms (Sánchez-Criado et al., 2005a) and functional plasma membrane ERα (Sánchez-Criado et al., 2005b). Activation of all these ERs with the physiological dosage (Schuiling et al., 1984, 1987) of EB over six days reduced the effects of OVX on gonadotroph size, PR expression and RER, Golgi complex and secretory granules features.

As the cognate ligand EB, TX reduced the morphological effects of OVX-induced E_2 withdrawal by inducing shrinkage, reorganization of membrane-enclosed intracellular organelles and expression of PR in gonadectomy cells. However, these effects of TX were of higher magnitude than those exerted by EB. The SERM TX is a type I oestrogen receptor agonist/antagonist (Smith and O'Malley, 2004) which, at the rat pituitary level, exhibits high affinity for nuclear ERα (Sánchez-Criado et al., 2005b; Alonso et al., 2006; Garrido-Gracia et al., 2007). Thus, in the absence of E2, TX induces ERα expression in the gonadotroph (Sánchez-Criado et al., 2005a), and ERα effects similar to those elicited by the selective agonist PPT: negative feedback on LH secretion, PR expression and PR-dependent (Chappell et al., 1999) LHRH self-priming (Sánchez-Criado et al., 2002, 2004, 2006a; Bellido et al., 2003). Moreover, the effects of TX on LH secretion in OVX rats are blocked by the selective ERα antagonist methyl-piperidino-pyrazole (Garrido-Gracia et al., 2007).

The simultaneous administration of TX and the selective ERß agonist DPN had morphological effects somehow similar to those of EB alone, which suggests an inhibitory action of ERß upon nuclear ERα. The effects of DPN alone on long-term OVX rats only mimic those of ERα activation (Sánchez-Criado et al., 2006a): shrinkage of hypertrophied gonadotrophs, an increase of gonadotrophs expressing PR and an increase in PR protein. On the contrary, DPN administered to TX-treated OVX rats reduced TX-induced shrinkage, reorganization of membrane-enclosed intracellular organelles, and PR expression in gonadectomy cells. Similarly, administration of EB alone induces full physiological (Sánchez-Criado et al., 2004) and morphological (present results) oestrogenic effects in gonadectomy cells. However, EB had antagonist-like actions when administered to TX-treated OVX rats. One explanation is that TX, at the pharmacological dosage used, saturates mainly nuclear ERα (Sánchez-Criado et al., 2005a, 2005b, 2006a, 2006b; Garrido-Gracia et al., 2007). In this way, the binding of E2 to gonadotrope ERs in TX-treated rats would be restricted to both nuclear ERß, whose action would be inhibitory of ERα activation (Sánchez-Criado et al., 2005b, Alonso et al., 2006), and surface ERα, which would have no effect on gonadotroph morphology and PR expression (present results). ERß appears to antagonize the stimulatory actions of ERα in many other contexts (Paech et al., 1997; Hall and McDonnell, 1999; McDonnell et al., 2002; Weihua et al., 2003; Koehler et al., 2005) including the mammary gland, (Paruthiyil et al., 2004; Helguero et al., 2005), bone (Lindberg et al., 2003), uterus (Weihua et al., 2000; Frasor et al., 2003) and hair follicles (Ohnemus et al., 2005) in mice.

The inhibitory action of ERß on nuclear ERα actions is of relevance regarding the biology of PR in the gonadotroph, because expression of ERα-dependent PR in the rat gonadotroph is critical for the physiological preovulatory LH release (Brown and Naftolin, 1972; Attardi, 1984; Turgeon and Waring, 1990; Levine, 1997; Cenni and Picard, 1999). In OVX rats, both shrinkage of hypertrophied gonadotrophs and reorganization of membrane-enclosed intracellular organelles, where assembly and posttranslational modifications of LH chains take place, precede the acquisition of the

gonadotroph ability to express PR (Sánchez-Criado et al., 2006a). Results showed an inverse relationship between gonadotroph size and expression of nuclear PR not related to the ligand treatment employed. Thus, gonadotrophs smaller than 150 μm^2 in area (subtypes III and IV) always expressed PR, while those greater than 250 μm^2 (subtypes I and II) did not. In this manner, a cut-off of about 200 μm^2 in area, when cytoplasmic vesiculation begins to appear in the gonadotroph after OVX (transition from subtype III to subtype II), seemed to be the size threshold to lose the ability to express PR and, consequently, full responsiveness to LHRH (Sánchez-Criado et al., 2005a), the PR-dependent component of the LH surge (Chappell et al., 1999).

The selective ERα agonist PPT had no effect on gonadotroph morphology when administered to TX-treated rats, which suggests that extranuclear ERα does not modify the effects of nuclear ERα activation with TX in gonadectomy cells. Because of the high selectivity of PPT for the ERα isoform and its actions (Stauffer et al., 2000; Sánchez-Criado et al., 2004, 2006a), the results may, in addition, demonstrate a competition between PPT and TX for the same ER pool. Since PPT had no effect on TX agonistic action, the results are also indicative that the inhibitory effects of EB on TX agonistic action were not ERα-mediated; a fact also evidenced in DPN injected rats. Since TX has very low affinity for membrane ERα in the gonadotroph (Sánchez-Criado et al., 2005b) and PPT is able to activate membrane ERα in long-term OVX rats (Sánchez-Criado et al., 2004), the lack of effect of PPT on gonadotroph morphology in TX-treated rats indicates that surface ERα was not involved in the E_2 reversion of OVX-induced morphological changes. The TX-elicited LHRH self-priming through nuclear ERα (Sánchez-Criado et al., 2002) is blocked by activation of membrane ERα, either with EB, PPT or E2-BSA through membrane-initiated signaling (Sánchez-Criado et al., 2005b). Hence, though activation of surface ERα in the gonadotroph is not involved in the reversal effects of E_2 on the OVX gonadotroph morphology, they play a relevant modulatory role in the PR-dependent LH secretion (Sánchez-Criado et al., 2006b; Garrido-Gracia et al., 2007).

In conclusion, the reorganization of membrane-enclosed intracellular organelles and the shrinkage that precedes the expression of PR induced by E_2 in gonadectomy cells of the OVX rat were due to activation of both nuclear ER isoforms, while membrane ERα had no effect. Thus, activation of nuclear ERα would be a determinant for the reversal effects of E_2 on gonadotroph morphology and PR expression, and the simultaneous activation of ERß would modulate the action of nuclear ERα in an inhibitory fashion.

Acknowledgements. Funding was provided by grants BFU2005-01441 and AGL2006-09016/GAN from the DGICYT (Spain) to JES-C and JMM, respectively.

References

Aguilar R., Bellido C., Garrido-Gracia J.C., Alonso R. and Sánchez-Criado J.E. (2006). Estradiol and its membrane-impermeable conjugate estradiol-BSA inhibit tamoxifen-stimulated prolactin secretion in incubated rat pituitaries. Reproduction 131, 763-769.

Alonso R., Marín F., González M., Guelmes P., Bellido C., Hernández G., Marín R., Díaz M. and Sánchez-Criado J.E. (2006). The hypothalamus-pituitary-ovarian axis as a model system for the study of SERM effects: An overview of experimental and clinical studies. In: Selective Estrogen Receptor Modulators. New Brand of Multitarget Drugs. Cano A., Calaf i Alsina J. and Dueñas-Diez J.L. (eds). Springer. Berlin. pp 103-139.

Arreguin-Arevalo J.L., Davis T.L. and Nett T.M. (2007). Differential modulation of gonadotropin secretion by selective estrogen receptor 1 and estrogen receptor 2 agonists in ovariectomized ewes. Biol. Reprod. 77, 320-328.

Attardi B. (1984). Progesterone modulation of the luteinizing hormone surge: regulation of hypothalamic and pituitary progestin receptors. Endocrinology 115, 2113-2122.

Bellido C., Martín de las Mulas J., Tena-Sempere M., Aguilar R., Alonso R. and Sánchez-Criado J.E. (2003). Tamoxifen induces gonadotropin-releasing hormone self-priming through an estrogen-dependent progesterone receptor expression in the gonadotrope of the rat. Neuroendocrinology 77, 424-434.

Blanco A., Agüera E., Flores R., Artacho-Pérula E. and Monterde J.G. (2001). Morphological and quantitative study of the Leydig cells of pigs fed with anabolic doses of clenbuterol. Res. Vet. Sci. 71, 85-91.

Brown-Grant K. and Naftolin F. (1972). Facilitation of luteinizing hormone secretion in the female rat by progesterone. J. Endocrinol. 53, 37-46.

Castro-Vazquez A. and McCann S.M. (1975). Cyclic variation in the increased responssiveness of the pituitary to luteinizing hormone-releasing hormone (LHRH) induced by LHRH. Endocrinology 97, 13-19.

Cenni B. and Picard D. (1999). Ligand-independent activation of steroid receptors: new role for old players. TEM 10, 41-46.

Chappell P.E., Schneider J.S., Kim P., Xu M., Lydon J.P., O'Malley B.W. and Levine J.E. (1999). Absence of gonadotropin surges and gonadotropin-releasing hormone self-priming in ovariectomized (OVX), estrogen (E2)-treated, progesterone receptor knockout (PRKO) mice. Endocrinology 140, 3653-3658.

Childs G.V. (1986). Functional ultrastructure of gonadotropes: a review. In: Current topics in neuroendocrinology. Morphology of hypothalamus and its connections. Ganten D. and Pfaff D. (eds). Springer-Verlag. Berlin. pp 49-97.

Childs G.V., Ellison D.G., Lorenzen J.R., Collins T.H. and Schwartz N.B. (1982). Immunocytochemical studies of gonadotropin storage in developing castration cells. Endocrinology 111, 1318-1328.

Cornea A., Janovick J.A., Stanislaus D. and Conn P.M. (1998). Redistribution of G(q/11) alpha in the pituitary gonadotrope in response to a gonadotropin-releasing hormone agonist. Endocrinology 139, 397-402.

Fellmann D., Bresson J.L., Clavequin M.C. and Bugnon C. (1982). Quantitative immunocytochemical studies on the gonadotrophs isolated from the pituitary of the male rat. Cell. Tissue Res. 224, 137-144.

Fink G. (1988). Gonadotropin secretion and its control. In: The Physiology of Reproduction. Knobil E. and Neill J. (eds). Raven

Press. New York. pp 1349-1377.

Fink G. (1995). The self-priming effect of LHRH: a unique servomechanism and possible cellular model for memory. Front. Neuroendocrinol. 16, 83-190.

Fox S.R., Harlan R.E., Shivers B.F. and Pfaff D.W. (1990). Chemical characterization of neuroendocrine target of progesterone in the female rat brain and pituitary. Neuroendocrinology 51, 276-283.

Frasor J., Barnett D.H., Danes J.M., Hess R., Parlow A.F. and Katzenellenbogen B.S. (2003). Response-specific and ligand dose-dependent modulation of estrogen receptor (ER) alpha activity by ERbeta in the uterus. Endocrinology 144, 3159-3166.

Freeman M.E. (1988). The ovarian cycle of the rat. In: The Physiology of reproduction. Knobil E. and Neill J. (eds). Raven Press. New York. pp 1893-1928.

Garner L.L. and Blake C.A. (1981). Ultrastructural, immunocytochemical study of the LH secreting cell of the rat anterior pituitary gland: changes occurring after ovariectomy. Biol. Reprod. 24, 461-474.

Garrido-Gracia J.C., Gordon A., Bellido C., Aguilar R., Barranco I., Millán Y., Martín de las Mulas J. and Sánchez-Criado J.E. (2007). The integrated action of oestrogen receptor isoforms and sites with progesterone receptor in the gonadotrope modulates LH secretion: evidence from tamoxifen-treated ovariectomized rats. J. Endocrinol. 193, 107-119.

Gartner J.P. and Hiatt J.L. (2001). Endocrine system. In: Color textbook of histology. 2nd ed. W.B. Saunders co. Philadelphia. pp 301-324.

Genbacev O. and Pantic V. (1975). Pituitary cell activities in gonadectomized rats treated with estrogen. Cell Tissue Res. 157, 273-282.

Hall J.M. and McDonnell D.P. (1999). The estrogen receptor ß-isoform (ERß of the human estrogen receptor modulates ERα transcriptional activity and is a key regulator of cellular response to estrogen and antiestrogens. Endocrinology 140, 5566-5578.

Helguero L.A., Faulds M.H., Gustafsson J.A. and Haldosén L.A. (2005). Estrogen receptor alfa (ERα) and beta (ERß) differentially regulate proliferation and apoptosis of the normal mammary epithelial cell line HC11. Oncogene 24, 6605-6616

Koehler K.F., Helguero L.A., Haldosén L.A., Warner M. and Gustafsson J.A. (2005). Reflections on the discovery and significance of estrogen receptor ß. Endocr. Rev. 26, 465-478.

Legan S.J. and Tsai H.W. (2003). Oestrogen receptor-α and ß-immunoreactivity in gonadotropin-releasing hormone neurones after ovariectomy and chronic exposure to oestradiol. J. Neuroendocrinol. 15, 1164-1179.

Levin E.R. (2005). Integration of the extranuclear and nuclear actions of estrogen. Mol. Endocrinol. 19, 1951-1959.

Levine J.E. (1997). New concepts of the neuroendocrine regulation of gonadotropin surges in rats. Biol. Reprod. 56, 293-302.

Lindberg M.K., Moverare S., Skrtic S., Gao H., Dahlman-Wright K., Gustafsson J.A. and Ohlsson C. (2003). Estrogen receptor (ER)-beta reduces ER alpha-regulated gene transcrition, supporting a "ying yang" relationship between ERalpha and ERbeta in mice. Mol. Endocrinol. 17, 203-208.

McDonnell D.P., Wijayaratne A., Chang C.Y. and Norris J.D. (2002). Elucidation of the molecular mechanism of action of selective estrogen receptor modulators. American. J. Cardiol. 90, 35F-43F.

Meyers M.J., Sun J., Carlson K.E., Marriner A., Katzenellenbogen B.S. and Katzenellenbogen J.A. (2001). Estrogen receptor-ß potency-selective ligands: structure-activity relationship studies of diarylpropionitriles and their acetylene and polar analogues. J. Med. Chem. 44, 4230-4251.

Moriarty G.C. (1975). Electron microscopic-immunocytochemical studies of rat pituitary gonadotrophs: A sex difference in morphology and cytochemistry of LH cells. Endocrinology 97, 1215-1225.

Morris J.F., Lewis C.E. and Fink G. (1986). LH-RH priming in gonadotrophs: A model system for the analysis of neuroendocrine mechanism at the cellular level. In: Neuroendocrine molecular biology. Fink G., Harmar A.J. and McKerns K.W. (eds). Plenum Press. New York. pp 341-352.

Nishihara E., Nagayama Y., Inoue S., Hiroi H., Muramatsu M., Yamashita S. and Koji T. (2000). Ontogenic changes in the expression of estrogen receptor α and ß in rat pituitary gland detected by immunohistochemistry. Endocrinology 141, 615-620.

Ohnemus U., Uenalan M., Conrad F., Handjiski B., Mecklenburg L., Nakamura M., Izunza J., Gustafsson J.A. and Paus R.. (2005). Hair cycle control by estrogens: catagen induction via estrogen receptor (ER)a· is checked by ER, signaling. Endocrinology 146, 1214-1225.

Paech K., Well P., Kuiper G.G.J.M., Nilsson S., Gustafsson J.A., Kushner P.J. and Scanlan T.S. (1997). Differential ligand acrtivation of estrogen receptors ERα and ERß at AP1 sites. Science 277, 1508-1510.

Paruthiyil S., Parmar H., Kerekatte V., Cunha G.R., Firestone G.L. and Leitman D.C. (2004). Estrogen receptor beta inhibits human breast cancer cell prolifaration and tumor formation by causing G2 cell cycle arrest. Cancer Res. 64, 423-428.

Pelletier G., Li S., Phaneuf D., Martel C. and Labrie F. (2003). Morphological studies of prolactin-secreting cells in estrogen receptor a and estrogen receptor ß knockout mice. Neuroendocrinology 77, 324-333.

Petersson K. and Gustafsson J.A. (2001). Role of estrogen receptor , in estrogen action. Ann. Rev. Physiol. 63, 165-192.

Pierce J.G. (1988). Gonadotropins: Chemistry and biosynthesis. In: The physiology of reproduction. Knobil E. and Neill J. (eds). Raven Press. New York. pp 1335-1348.

Sánchez-Criado J.E., Guelmes P., Bellido C., González M., Hernández G., Aguilar R., Garrido-Gracia J.C., Bello A.R. and Alonso R. (2002). Tamoxifen but not other selective estrogen receptor modulators antagonizes estrogen actions on luteinizing hormone secretion while inducing gonadotropin-releasing hormone self-priming in the rat. Neuroendocrinology 76, 203-213.

Sánchez-Criado J.E., Martín de las Mulas J., Bellido C., Tena-Sempere M., Aguilar R. and Blanco A. (2004). Biological role of pituitary estrogen receptors ERα and ER, on progesterone receptor expression and action and on gonadotropin and prolactin secretion in the rat. Neuroendocrinology 79, 247-258.

Sánchez-Criado J.E., Martín de las Mulas J., Bellido C., Aguilar R. and Garrido-Gracia J.C. (2005a). Gonadotrope oestrogen receptor-a and ß, and progesterone receptor immunoreactivity after ovariectomy and exposure to oestradiol benzoate, tamoxifen or raloxifene in the rat: correlation with LH secretion. J. Endocrinol. 184, 59-68.

Sánchez-Criado J.E., Bellido C., Aguilar R. and Garrido-Gracia J.C. (2005b). A paradoxical inhibitory effect of oestradiol-17ß on GnRH self-priming in pituitaries from tamoxifen-treated rats. J. Endocrinol. 186, 43-49.

Sánchez-Criado J.E., Martín de las Mulas J., Bellido C., Navarro V.M., Aguilar R., Garrido-Gracia J.C., Malagón M.M., Tena-Sempere M. and Blanco A. (2006a). Gonadotropin-secreting cells in ovariectomized rats treated with different oestrogen receptor ligands: a modulatory role for ER, in the gonadotrope? J. Endocrinol.

188, 167-177.

Sánchez-Criado J.E., Garrido-Gracia J.C., Bellido C., Aguilar R., Guelmes P., Abreu P., Alonso R., Barranco I., Millán Y. and Martín de las Mullas J. (2006b). Oestradiol-17, inhibits tamoxifen-induced LHRH self-priming blocking hormone dependent and ligand-independent activation of the gonadotrope progesterone receptor in the rat. J. Endocrinol. 190, 73-84.

Schuiling G.A., Pols-Valkhof N., van der Schaaf-Verdonk G.C.J. and Koiter T.R. (1984). Blockade of LH and FSH secretion by LH-releasing hormone, by the LH releasing hormone analogue, buserelin, and by the combined treatment with LH-releasing hormone and oestradiol benzoate. J. Endocrinol. 103, 301-309.

Schuiling G.A., Pols-Valkhof N., Moes H. and Koiter T.R. (1987). Plasma gonadotrophin concentrations, pituitary gonadotrophin content and pituitary responsiveness to LHRH in rats treated with LHRH and oestradiol benzoate. J. Endocrinol. 115, 469-475.

Scott R.E., Wu-Peng X.S. and Pfaff D.W. (2002). Regulation and espression of progesterone receptor mRNA isoforms A and B in the male and female rat hypothalamus and pituitary following oestrogen treatment. J. Neuroendocrinol. 14, 175-183.

Smith M.S., Freeman M.E. and Neill J.D. (1975). The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat: prolactin, gonadotropin and steroid levels associated with rescue of corpus luteum of pseudopregnancy. Endocrinology 96, 219-226.

Smith P.J. and O'Malley B.W. (2004). Coregulator function: a key to understanding tissue specificity of selective receptor modulators. Endocr. Rev. 25, 45-71.

Stauffer S.R., Coletta C.J., Tedesco R., Nishiguchi G., Carlson K., Sun J., Katzenellenbogen B.S. and Katzenellenbogen J.A. (2000). Pyrazole ligands: structure-affinity/activity relationships and estrogen receptor-α-selective agonist. J. Med. Chem. 43, 4934-4947.

Sun J., Huang Y.R., Harrington W.R., Sheng S., Katzenellenbogen J.A. and Katzenellenbogen B.S. (2002). Antagonist selective for estrogen receptorα. Endocrinology 143, 941-947.

Tougard C.L. and Tixier-Vidal A. (1988). Lactotropes and gonadotropes. In: The physiology of reproduction. Knobil E. and Neill J. (eds). Raven Press. New York. pp 1305-1333.

Turgeon J.L. and Waring D.W. (1990). Rapid augmentation by progesterone of agonist-stimulated luteinizing hormone secretion by cultured pituitary cells. Endocrinology 127, 773-780.

Tzuckerman M.T., Esty A., Santiso-Mere D., Danielian P., Parker M.G., Stein R.B., Pike J. and McDonnell D.P. (1994). Human estrogen receptor transcriptional capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions. Mol. Endocrinol. 8, 21-30.

Weihua Z., Andersson S., Cheng G., Simpson E.R., Warner M. and Gustafsson J.A. (2003). Update on estrogen signaling. FEBS Let. 546, 17-24.

Weihua Z., Saji S., Mäkinen S., Cheng G., Jensen E.V., Warner M. and Gustafsson J.A. (2000). Estrogen receptor (ER)α, a modulator of ERα in the uterus. Proc. Natl. Acad. Sci. USA 97, 5936-5941.

Accepted June 18, 2008